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REGULATION OF GENE EXPRESSION IN PLANTS

This invention relates to methods of modulating the expression of desired genes in plants, and to DNA
5   sequences and genetic constructs for use in these methods. In particular, the invention relates to methods and constructs for targeting of expression specifically to the endosperm of the seeds of cereal plants such as wheat, and for modulating the time of expression in the target tissue.
10 This is achieved by the use of promoter sequences from enzymes of the starch biosynthetic pathway. In a preferred embodiment of the invention, the sequences and/or promoters are those of starch branching enzyme I, starch branching enzyme II, soluble starch synthase I, and starch debranching
15 enzyme, all derived from *Triticum tauschii*, the D genome donor of hexaploid bread wheat.

A further preferred embodiment relates to a method of identifying variations in the characteristics of plants.

20 BACKGROUND OF THE INVENTION

Starch is an important constituent of cereal grains and of flours, accounting for about 65-67% of the weight of the grain at maturity. It is produced in the amyloplast of the grain endosperm by the concerted action of
25 a number of enzymes, including ADP-Glucose pyrophosphorylase (EC 2.7.7.27), starch synthases (EC 2.4.1.21), branching enzymes (EC 2.4.1.18) and debranching enzymes (EC 3.2.1.41 and EC 3.2.1.68) (Ball *et al*, 1996; Martin and Smith, 1995; Morell *et al*, 1995). Some of the proteins involved in the
30 synthesis of starch can be recovered from the starch granule (Denyer *et al*, 1995; Rahman *et al*, 1995).

Most wheat cultivars normally produce starch containing 25% amylose and 75% amylopectin. Amylose is composed of large linear chains of α (1-4) linked α-D-
35 glucopyranosyl residues, whereas amylopectin is a branching form of α-glycan linked by α (1-6) linkages. The ratio of amylose and amylopectin, the branch chain length and the

number of branch chains of amylopectin are the major factors
which determine the properties of wheat starch.

Starch with various properties has been widely
used in industry, food science and medical science. High
amylose wheat can be used for plastic substitutes and in
paper manufacture to protect the environment; in health
foods to reduce bowel cancer and heart disease; and in
sports foods to improve the athletes' performance. High
amylopectin wheat may be suitable for Japanese noodles, and
is used as a thickener in the food industry.

Wheat contains three sets of chromosomes (A, B and
D) in its very large genome of about 10^{10} base pairs (bp).
The donor of the D genome to wheat is *Triticum tauschii*, and
by using a suitable accession of this species the genes from
the D genome can be studied separately (Lagudah et al,
1991).

There is comparatively little variation in starch
structure found in wheat varieties, because the hexaploid
nature of wheat prevents mutations from being readily
identified. Dramatic alterations in starch structure are
expected to require the combination of homozygous recessive
alleles from each of the 3 wheat genomes, A, B and D. This
requirement renders the probability of finding such mutants
in natural or mutagenised populations of wheat very low.
Variation in wheat starch is desirable in order to enable
better tailoring of wheat starches for processing and end-
user requirements.

Key commercial targets for the manipulation of
starch biosynthesis are:

1.   "Waxy" wheats in which amylose content is
decreased to insignificant levels. This outcome is expected
to be obtained by eliminating granule-bound starch synthase
activity.

2.   High amylose wheats, expected to be obtained
by suppressing starch branching enzyme-II activity.

3.   Wheats which continue to synthesise starch
at elevated temperatures, expected to be obtained by

identifying or introducing a gene encoding a heat-stable
soluble starch synthase.

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        4.   "Sugary types" of wheat which contain
increased amylose content and free sugars, expected to be
obtained by manipulating an isoamylase-type debranching
enzyme.

        There are two general strategies which may be used
to obtain wheats with altered starch structure:

        (a)   using genetic engineering strategies to
suppress the activity of a specific gene, or to introduce a
novel gene into a wheat line; and

        (b) selecting among existing variation in wheat for
        missing ("null") or altered alleles of a gene in
        each of the genomes of wheat, and combining
        these by plant breeding.

However, in view of the complexity of the gene families,
particularly starch branching enzyme I (SBE I), without the
ability to target regions which are unique to genes
expressed in endosperm, modification of wheat by combination

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of null alleles of several enzymes in general represents an
almost impossible task.

        Branching enzymes are involved in the production
of glucose α-1,6 branches. Of the two main constituents of
starch, amylose is essentially linear, but amylopectin is

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highly branched; thus branching enzymes are thought to be
directly involved in the synthesis of amylopectin but not
amylose. There are two types of branching enzymes in plants
, starch branching enzyme I (SBE I) and starch branching
enzyme II (SBE II), and both are about 85 kDa in size. At

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the nucleic acid level there is about 65% sequence identity
between types I and II in the central portion of the
molecules; the sequence identity between SBE I from
different cereals is about 85% overall (Burton *et al*, 1995;
Morell *et al*, 1995).

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        In cereals, SBE I genes have so far been reported
only for rice (Kawasaki *et al*, 1991; Rahman *et al*, 1997). A
cDNA sequence for wheat SBE I is available on the GenBank

database (Accession No. Y12320; Repellin A., Nair R.B., Baga M., and Chibbar R.N.: Plant Gene Register PGR97-094, 1997). As far as we are aware, no promoter sequence for wheat SBE I has been reported.

5   We have characterised an SBE I gene, designated *wSBE I-D2*, from *Triticum tauschii*, the donor of the D genome to wheat (Rahman *et al*, 1997). This gene encoded a protein sequence which had a deletion of approximately 65 amino acids at the C-terminal end, and appeared not to contain

10   some of the conserved amino acid motifs characteristic of this class of enzyme (Svensson, 1994). Although *wSBE I-D2* was expressed as mRNA, no corresponding protein has yet been found in our analysis of SBE I isoforms from the endosperm, and thus it is possible that this gene is a transcribed

15   pseudogene.

Genes for SBE II are less well characterised; no genomic sequences are available, although SBE II cDNAs from rice (Mizuno *et al*, 1993; Accession No. D16201) and maize (Fisher *et al*, 1993; Accession No. L08065) have been

20   reported. In addition, a cDNA sequence for SBE II from wheat is available on the GenBank database (Nair *et al*, 1997; Accession No. Y11282); although the sequences are very similar to those reported herein, there are differences near the N-terminal of the protein, which specifies its

25   intracellular location. No promoter sequences have been reported, as far as we are aware.

Wheat granule-bound starch synthase (GBSS) is responsible for amylose synthesis, while wheat branching enzymes together with soluble starch synthases are

30   considered to be directly involved in amylopectin biosynthesis. A number of isoforms of soluble and granule-bound starch synthases have been identified in developing wheat endosperm (Denyer *et al*, 1995). There are three distinct isoforms of starch synthases, 60 kDa, 75-77 kDa and

35   100-105 kDa, which exist in the starch granules (Denyer *et al*, 1995; Rahman *et al*, 1995). The 60 kDa GBSS is the product of the wx gene. The 75-77 kDa protein is a wheat

soluble starch synthase I (SSSI) which is present in both the soluble fraction and the starch granule-bound fraction of the endosperm. However, the 100-105 kDa proteins, which are another type of soluble starch synthase, are located

5   only in starch granules (Denyer et al, 1995; Rahman et al, 1995). To our knowledge there has been no report of any complete wheat SSS I sequence, either at the protein or the nucleotide level.

Both cDNA and genomic DNA encoding a soluble

10   starch synthase I of rice have been cloned and analysed (Baba et al, 1993; Tanaka et al, 1995). The cDNAs encoding potato soluble starch synthase SSSII and SSSIII and pea soluble starch synthase SSSII have also been reported (Edwards et al, 1995; Marshall et al, 1996; Dry et al,

15   1992). However, corresponding full length cDNA sequences for wheat have hitherto not been available, although a partial cDNA sequence (Accession No. U48227) has been released to the GenBank database.

Approach (b) referred to above has been

20   demonstrated for the gene for granule-bound starch synthase. Null alleles on chromosomes 7A, 7D and 4A were identified by the analysis of GBSS protein bands by electrophoresis, and combined by plant breeding to produce a wheat line containing no GBSS, and no amylose (Nakamura et al, 1995).

25   Subsequently, PCR-based DNA markers have been identified, which also identify null alleles for the GBSS loci on each of the three wheat genomes. Despite the availability of a considerable amount of information in the prior art, major problems remain. Firstly, the presence of three separate

30   sets of chromosomes in wheat makes genetic analysis in this species extraordinarily complex. This is further complicated by the fact that a number of enzymes are involved in starch synthesis, and each of these enzymes is itself present in a number of forms, and in a number of

35   locations within the plant cell. Little, if any, information has been available as to which specific form of each enzyme is expressed in endosperm. For wheat, a limited

amount of nucleic acid sequence information is available,
but this is only cDNA sequence; no genomic sequence, and
consequently no information regarding promoters and other
control sequences, is available. Without being able to
5   demonstrate that the endosperm-specific gene within a family
has been isolated, such sequence information is of limited
practical usefulness.

SUMMARY OF THE INVENTION

10   In this application we report the isolation and
identification of novel genes from *T. tauschii*, the D-genome
donor of wheat, that encode SBE I, SBE II, a 75 kDa SSS I,
and an isoamylase-type debranching enzyme (DBE). Because of
the very close relationship between *T. tauschii* and wheat,
15   as discussed above, results obtained with *T. tauschii* can be
directly applied to wheat with little if any modification.
Such modification as may be required represents routine
trial and error experimentation. Sequences from these genes
can be used as probes to identify null or altered alleles in
20   wheat, which can then be used in plant breeding programmes
to provide modifications of starch characteristics. The
novel sequences of the invention can be used in genetic
engineering strategies or to introduce a desired gene into a
host plant, to provide antisense sequences for suppression
25   of one or more specific genes in a host plant, in order to
modify the characteristics of starch produced by the plant.
          By using *T. tauschii*, we have been able to examine
a single genome, rather than three as in wheat, and to
identify and isolate the forms of the starch synthesis genes
30   which are expressed in endosperm. By addressing genomic
sequences we have been able to isolate tissue-specific
promoters for the relevant genes, which provides a mechanism
for simultaneous manipulation of a number of genes in the
endosperm. Because *T. tauschii* is so closely related to
35   wheat, results obtained with this model system are directly
applicable to wheat, and we have confirmed this
experimentally. The genomic sequences which we have

determined can also be used as probes for the identification and isolation of corresponding sequences, including promoter sequences, from other cereal plant species.

In its most general aspect, the invention provides
5   a nucleic acid sequence encoding an enzyme of the starch biosynthetic pathway in a cereal plant, said enzyme being selected from the group consisting of starch branching enzyme I, starch branching enzyme II, starch soluble synthase I, and debranching enzyme, with the proviso that
10 the enzyme is not soluble starch synthase I of rice, or starch branching enzyme I of rice or maize, and that starch branching enzyme II does not have the N-terminal amino acid sequence:

15   AASPGKVLVPDGEDDLASPA.

Preferably the nucleic acid sequence is a DNA sequence, and may be genomic DNA or cDNA. Preferably the sequence is one which is functional in wheat. More
20 preferably the sequence is derived from a *Triticum* species, most preferably *Triticum tauschii*.

Where the sequence encodes soluble starch synthase, preferably the sequence encodes the 75 kD soluble starch synthase of wheat.

25   Biologically-active untranslated control sequences of genomic DNA are also within the scope of the invention. Thus the invention also provides the promoter of an enzyme as defined above.

In a preferred embodiment of this aspect of the
30 invention, there is provided a nucleic acid construct comprising a nucleic acid sequence of the invention, a biologically-active fragment thereof, or a fragment thereof encoding a biologically-active fragment of an enzyme as defined above, operably linked to one or more nucleic acid
35 sequences facilitating expression of said enzyme in a plant, preferably a cereal plant. The construct may be a plasmid or a vector, preferably one suitable for use in the transformation of a plant. A particularly suitable vector is a bacterium of the genus *Agrobacterium*, preferably

Agrobacterium tumefaciens. Methods of transforming cereal plants using *Agrobacterium tumefaciens* are known; see for example Australian Patent No. 667939 by Japan Tobacco Inc.,

International Patent Application Number PCT/US97/10621 by Monsanto Company and Tingay et al (1997).

In a second aspect, the invention provides a nucleic acid construct for targeting of a desired gene to

5   endosperm of a cereal plant, and/or for modulating the time of expression of a desired gene in endosperm of a cereal plant, comprising one or more promoter sequences selected from SBE I promoter, SBE II promoter, SSS I promoter, and DBE promoter, operatively linked to a nucleic acid sequence

10   encoding a desired protein, and optionally also operatively linked to one or more additional targeting sequences and/or one or more 3' untranslated sequences.

The nucleic acid encoding the desired protein may be in either the sense orientation or in the antisense

15   orientation. Preferably the desired protein is an enzyme of the starch biosynthetic pathway. For example, the antisense sequences of GBSS, starch debranching enzyme, SBE II, low molecular weight glutenin, or grain softness protein I, may be used. Preferred sequences for use in sense orientation

20   include those of bacterial isoamylase, bacterial glycogen synthase, or wheat high molecular weight glutenin Bx17. It is contemplated that any desired protein which is encoded by a gene which is capable of being expressed in the endosperm of a cereal plant is suitable for use in the invention.

25   In a third aspect, the invention provides a method of modifying the characteristics of starch produced by a plant, comprising the step of:

(a)   introducing a gene encoding a desired enzyme of the starch biosynthetic pathway into a host plant, and/or

30   (b)   introducing an anti-sense nucleic acid sequence directed to a gene encoding an enzyme of the starch biosynthetic pathway into a host plant,

wherein said enzymes are as defined above.

Where both steps (a) and (b) are used, the enzymes

35   in the two steps are different.

Preferably the plant is a cereal plant, more preferably wheat or barley.

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As is well known in the art, anti-sense sequences can be used to suppress expression of the protein to which the anti-sense sequence is complementary. It will be evident to the person skilled in the art that different
5   combinations of sense and anti-sense sequences may be chosen so as to effect a variety of different modifications of the characteristics of the starch produced by the plant.

In a fourth aspect, the invention provides a method of targeting expression of a desired gene to the
10 endosperm of a cereal plant, comprising the step of transforming the plant with a construct according to the invention.

According to a fifth aspect, the invention provides a method of modulating the time of expression of a
15 desired gene in endosperm of a cereal plant, comprising the step of transforming the plant with a construct according to the second aspect of the invention.

Where expression at an early stage following anthesis is desired, the construct preferably comprises the
20 SBE II, SSS I or DBE promoters. Where expression at a later stage following anthesis is desired, the construct preferably comprises the SBE I promoter.

While the invention is described in detail in relation to wheat, it will be clearly understood that it is
25 also applicable to other cereal plants of the family Gramineae, such as maize, barley and rice.

Methods for transformation of monocotyledonous plants such as wheat, maize, barley and rice and for regeneration of plants from protoplasts or immature plant
30 embryos are well known in the art. See for example Lazzeri et al, 1991; Jahne et al, 1991 and Wan and Lemaux, 1994 for barley; Wirtzens et al, 1997; Tingay et al, 1997; Canadian Patent Application No. 2092588 by Nehra; Australian Patent Application No. 61781/94 by National Research Council of
35 Canada, Australian Patent No. 667939 by Japan Tobacco Co, and International Patent Application Number PCT/US97/10621 by Monsanto Company.

The sequences of ADP glucose pyrophosphorylase
from barley (Australian Patent Application No. 65392/94),
starch debranching enzyme and its promoter from rice
(Japanese Patent Publication No. Kokai 6261787 and Japanese
5   Patent Publication No. Kokai 5317057), and starch
debranching enzyme from spinach and potato (Australian
Patent Application No. 44333/96) are all known.

Detailed Description of the Drawings

10   The invention will be described in detail by
reference only to the following non-limiting examples and to
the figures.

         Figure 1 shows the hybridisation of genomic clones
isolated from *T. tauschii*.

15   DNA was extracted from the different clones,
digested with *BamHI* and hybridised with the 5' end of the
maize SBE I cDNA. Lanes 1, 2, 3 and 4 correspond to DNA
from clones λE1, λE2, λE6 and λE7 respectively. Note that
clones λE1 and λE2 give identical patterns, the SBE I gene
20   in λE6 is a truncated form of that in λE1, and λE7 gives a
clearly different pattern.

         Figure 2 shows the hybridisation of DNA from
T. tauschii.

         DNA from *T. tauschii* was digested with *BamHI* and
25   the hybridisation pattern compared with DNA from λE1 and λE7
digested with the same enzyme. Fragment E1.1 (see Figure 3)
from λE1 was used as the probe; it contains some sequences
that are over 80% identical to sequences in E7.8.
Approximately 25 µg of *T. tauschii* DNA was electrophoresed
30   in lane 1, and 200 pg each of λE1 and λE7 in lanes 2 and 3,
respectively.

         Figure 3 shows the restriction maps of clone λE1
and λE7. The fragments obtained with EcoRI and BamHI are
indicated. The fragments sequenced from λE1 are E1.1, E1.2,
35   a part of E1.7 and a part of E1.5.

         Figure 4 shows the comparison of deduced amino
acid sequence of wSBE I-D4 cDNA with the deduced amino acid

sequence of rice SBE I (RSBE I; Nakamura *et al*, 1992), maize
SBE I (MSBE I; Baba *et al*, 1991), wSBE I-D2 type cDNA (D2
cDNA; Rahman *et al*, 1997), pea SBE II (PESBE II, homologous
to maize SBE I; Burton *et al*, 1995), and potato SBE I
5   (POSBE; Cangiano *et al*, 1993). The deduced amino acid
sequence of the wSBE I-D4 cDNA is denoted by "D4cDNA".
Residues present in at least three of the sequences are
identified in the consensus sequence in capitals.

       Figure 5 shows the intron-exon structure of
10   wSBE I-D4 compared to the corresponding structures of rice
SBE I (Kawasaki *et al*, 1993) and wSBE I-D2 (Rahman *et al*,
1997). The intron-exon structure of wSBE I-D4 is deduced by
comparison with the SBE I cDNA reported by Repellin *et al*
(1997).

15   The dark rectangles correspond to exons and the
light rectangles correspond to introns. The bars above the
structures indicate the percentage identity in sequence
between the indicated exons and introns of the relevant
genes. Note that intron 2 shares no significant sequence
20   identity and is not indicated.

       Figure 6 shows the nucleotide sequence of part of
wSBE I-D4, the amino acid sequence deduced from this
nucleotide sequence, and the N-terminal amino acid sequence
of the SBE I purified from the wheat endosperm (Morell *et
25   al*, 1997).

       Figure 7 shows the hybridisation of SBE I genomic
clones with the following probes,
       A.   wSBE I-D45 (derived from the 5' end of the
gene and including sequence from fragments E1.1 and E1.7),
30   and
       B.   wSBE I-D43 (derived from the 3' end of the
gene and containing sequences from fragment E1.5). For
panel A, the tracks 1-13 correspond to clones λE1, λE2, λE6,
λE7, λE9, λE14, λE22, λE27, Molecular weight markers, λE29,
35   λE30, λE31 and λE52. For panel B, tracks 1-12 correspond to
clones λE1, λE2, λE6, λE7, λE9, λE14, λE22, λE27, λE29,
λE30, λE31 and λE52. Note that clones λE7 and λE22 do not

hybridise to either of the probes and are wSBE I-D2 type
genes. Also note that clone λE30 contains a sequence
unrelated to SBE I. The size of the molecular weight
markers in kb is indicated. Clones λE7 and λE22 do
5   hybridise with a probe from E1.1. which is highly conserved
between wSBE I-D2 and wSBE I-D4.

        Figure 8 shows the alignment of cDNA clones to
obtain the sequence represented by wSBE I-D4 cDNA. BED4 and
BED5 were obtained from screening the cDNA library with
10   maize BEI (Baba et al, 1991). BED1, 2 and 3 were obtained
by RT-PCR using defined primers.

        Figure 9a shows the expression of Soluble Starch
Synthase I (SSS), Starch Branching Enzyme I (BE I) and
Starch Branching Enzyme II (BE II) mRNAs during endosperm
15   development.

        RNA was purified from leaves, florets prior to
anthesis, and endosperm of wheat cultivar Rosella grown in a
glasshouse, collected 5 to 8 days after anthesis, 10 to 15
days after anthesis and 18 to 22 days after anthesis, and
20   from the endosperm of wheat cultivar Rosella grown in the
field and collected 12, 15 and 18 days after anthesis
respectively. Equivalent amounts of RNA were
electrophoresed in each lane. The probes were from the
coding region of the SM2 SSS I cDNA (from nucleotide 1615 to
25   1919 of the SM2 cDNA sequence); wSBE I-D43C (see Table I),
which corresponds to the untranslated 3' end of wSBE I-D4
cDNA (E1 (3'; and the 5' region of SBE9 (SBE9 (5'),
corresponding to the region between nucleotides 743 to 1004
of Genbank sequence Y11282. No hybridisation to RNA
30   extracted from leaves or preanthesis florets was detected.

        Figure 9b shows the hybridisation of RNA from the
endosperm of the hexaploid T. aestivum cultivar "Gabo" with
the starch branching enzyme I gene. The probe, wSBEI-D43, is
defined in Table 1.

35   Figure 9c shows the hybridisation of RNA from the
endosperm of the hexaploid T. aestivum cultivar "Wyuna" with

the starch branching enzyme II gene. The probe, wSBE II-D13, is defined in Table 2.

Figure 9d shows the hybridisation of RNA from the endosperm of the hexaploid *T. aestivum* cultivar "Gabo" with
5   the SSS I gene. The probe spanned the region from nucleotides 2025 to 2497 of the SM2 cDNA sequence shown in SEQ ID No:11.

Figure 9e shows the hybridisation of RNA from the endosperm of the hexaploid *T. aestivum* cultivar "Gabo" with
10   the DBE I gene. The probe, a DBE3' 3'PCR fragment, extends from nucleotide position 281 to 1072 of the cDNA sequence in SEQ ID No:16.

Figure 9f shows the hybridisation of RNA from the endosperm of the hexaploid *T. aestivum* cultivar "Gabo" with
15   the wheat actin gene. The probe was a wheat actin DNA sequence generated by PCR from wheat endosperm cDNA using primers to conserved plant actin sequences.

Figure 9g shows the hybridisation of RNA from the endosperm of the hexaploid *T. aestivum* cultivar "Gabo" with
20   a probe containing wheat ribosomal RNA 26S and 18S fragments (plasmid pta250.2 from Dr Bryan Clarke, CSIRO Plant Industry).

Figure 9h shows the hybridisation of RNA from the hexaploid wheat cultivar "Gabo" with the DBE I probe
25   described in Figure 9e. Lane 1; leaf RNA; lane 2, pre-anthesis floret RNA; lane 3, RNA from endosperm harvested 12 days after anthesis.

Figure 10 shows the comparison of wSBE I-D4 (sr 427.res ck: 6,362,1 to 11,099) and rice SBE I genomic
30   sequence (d10838.em_pl ck: 3,071,1 to 11,700)(Kawasaki *et al*, 1993; Accession Number D10838) using the programs Compares and DotPlot (Devereaux *et al*, 1984). The programs used a window of 21 bases with a stringency of 14 to register a dot.
35   Figure 11 shows the hybridisation of wheat DNA from chromosome-engineered lines using the following probes:

A.   wSBE I-D45 (from the 5' end of the gene),

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B.   wSBE I-D43 (from the 3' end of the gene), and

C.   wSBE I-D4R (repetitive sequence approximately 600 bp 3' to the end of wSBE I-D4 sequence.
5   N7AT7B, no 7A chromosome, four copies of 7B chromosome; N7BT7D, no 7B chromosome, four copies of 7D chromosome; NTDT7A, no 7D chromosome, four copies of 7A chromosome. The chromosomal origin of hybridising bands is indicated.

10   Figure 12 shows the hybridisation of genomic clones F1, F2, F3 and F4 with the entire SBE-9 sequence. The DNA from the clones was purified and digested with either *Bam*HI or *Eco*RI, separated on agarose, blotted onto nitrocellulose and hybridised with labelled SBE-9 (a SBE II
15   type cDNA). The pattern of hybridising bands is different in the four isolates.

       Figure 13a shows the N-terminal sequence of purified SBE II from wheat endosperm as in Morell *et al*, (1997).

20   Figure 13b shows the deduced amino acid sequence from part of wSBE II-D1 that encodes the N-terminal sequence as described in Morell *et al*, (1997).

       Figure 14 shows the deduced exon-intron structure for a part of wSBE II-D1. The scale is marked in bases.
25   The dark rectangles are exons.

       Figure 15 shows the hybridisation of DNA from chromosome engineered lines of wheat (cultivar Chinese Spring) with a probe from nucleotides 550-850 from SBE-9. The band of approximately 2.2 kb is missing in the line in
30   which chromosome 2D is absent.

       T2BN2A: four copies of chromosome 2B, no copies of chromosome 2A;

       T2AN2B: four copies of chromosome 2A, no copies of chromosome 2B;

35   T2AN2D: four copies of chromosome 2A, no copies of chromosome 2D.

Figure 16 shows the N-terminal sequence of SSS I protein isolated from starch granules (Rahman et al, 1995) and deduced amino acid sequence of part of Sm2.

Figure 17 shows the hybridisation of genomic clones sg1, 3, 4, 6 and 11 with the cDNA clone (sm2) for SSS I. DNA was purified from indicated genomic clones, digested with BamHI or SacI and hybridised to sm2. Note that the hybridisation patterns for sg1, 3 and 4 are clearly different from each other.

Figure 18 shows a comparison of the intron/exon structures of the wheat and rice soluble starch synthase genomic sequences. The dark rectangles indicate exons and the light rectangles represent introns.

Figure 19 shows the hybridisation of DNA from chromosome engineered lines of wheat (cultivar Chinese Spring) digested with PvuII, with the sm2 probe.

N7AT7B: no 7A chromosome, four copies of 7B chromosome;

N7BT7D: no 7B chromosome, four copies of 7D chromosome;

N7DT7A: no 7D chromosome, four copies of 7A chromosome.

A band is missing in the N7BT7A line.

Figure 20a shows the DNA sequence of a portion of the wheat debranching enzyme (WDBE-1)PCR product. The PCR product was generated from wheat genomic DNA (cultivar Rosella) using primers based on sequences conserved in debranching enzymes from maize and rice.

Figure 20b shows a comparison of the nucleotide sequence of wheat debranching enzyme I (WDBE-I) PCR fragment (WHEAT.DNA) with the maize Sugary-1 sequence (SUGARY.DNA).

Figure 20c shows a comparison between the intron/exon structures of wheat debranching enzyme gene and the maize sugary-1 debranching enzyme gene.

Figure 21a shows the results of Southern blotting of T. tauschii DNA with wheat DBE-I PCR product. DNA from T. tauschii was digested with BamHI , electrophoresed,

blotted and hybridised to the wheat DBE-I PCR product
described in Figure 20a. A band of approximately 2 kb
hybridised.

Figure 21b shows Chinese Spring nullisomic/
5   tetrasomic lines probed with probes from the DBE gene. Panel
(I) shows hybridisation with a fragment spanning the region
from nucleotide 270 to 465 of the cDNA sequence shown in SEQ
ID No:16 from the central region of the DBE gene. Panel
(II) shows hybridisation with a probe from the 3' region of
10   the gene, from nucleotide 281 to 1072 of the cDNA sequence
given in SEQ ID No:16.

Figures 22a to 22e show diagrammatic
representations of the DNA vectors used for transient
expression analysis. In each of the sequences the N-terminal
15   methionine encoding ATG codon is shown in bold.

Figure 22a shows a DNA construct pwsssIpro1gfpNOT
containing a 1042 base pair region of the wheat soluble
starch synthase I promoter (wSSSIpro1, from -1042 to -1, SEQ
ID No:18) fused to the green fluorescent protein (GFP)
20   reporter gene.

Figure 22b shows a DNA construct pwsssIpro2gfpNOT
containing a 3914 base pair region of the wheat soluble
starch synthase I promoter (wSSSIpro2, from -3914 to -1, SEQ
ID No:18) fused to the green fluorescent protein (GFP)
25   reporter gene.

Figure 22c shows a DNA construct psbeIIpro1gfpNOT
containing an 1203 base pair region of the wheat starch
branching enzyme II promoter (sbeIIpro1, from 1 to 1023 SEQ
ID No:10 fused to the green fluorescent protein (GFP)
30   reporter gene.

Figure 22d shows a DNA construct psbeIIpro2gfpNOT
containing a 1353 base pair region of the wheat starch
branching enzyme II promoter and transit peptide coding
region (sbeIIpro2, regions 1-1203, 1204 to 1336 and 1664 to
35   1680 of SEQ ID No:10 fused to the green fluorescent protein
(GFP) reporter gene.

Figure 22e shows a DNA construct pact_jsgfg_nos

containing the plasmid backbone of pSP72 (Promega), the rice *ActI* actin promoter (McElroy et al. 1991), the GFP gene (Sheen et al. 1995) and the *Agrobacterium tumefaciens* nopaline synthase (nos) terminator (Bevan et al. 1983).

5   Figure 23 shows T DNA constructs for stable transformation of rice by *Agrobacterium*. The backbone for each plasmid is p35SH-iC (Wang et al 1997). The various promoter-GFP-Nos regions inserted are shown in (a), (b), (c) and (d) respectively, and are described in detail in Example

10   24. Each of these constructs was inserted into the NotI site of p35SH-iC using the NotI flanking sites at each end of the promoter-GFP-Nos regions. The constructs were named (a) p35SH-iC-BEIIpro1_GFP_Nos, (b) p35SH-iC-BEIIpro2_GFP_Nos (c) p35SH-iC-SSIpro1_GFP_Nos and (d) p35SH-iC-

15   SSIpro2_GFP_Nos

          Figure 24 illustrates the design of 15 intron-spanning BE II primer sets. Primers were based on wSBE II-D1 sequence (SEQ ID No:10), and were designed such that intron sequences in the wSBE II-D1 sequence (deduced

20   from Figure 13b and Nair *et al*, 1997; Accession No. Y11282) were amplified by PCR.

          Figure 25 shows the results of amplification using the SBE II-Intron 5 primer set (primer set 6: sr913F and WBE2E6 R) on various diploid, tetraploid and hexaploid

25   wheats.

          i) *T.boeodicum* (A genome diploid)
          ii) *T.tauschii* (D genome diploid)
          iii) *T.aestivum* cv. Chinese Spring ditelosomic line
               2AS (lacking chromosome arm 2AL)
30   iv) Crete 10 (AABB tetraploid)
          v) *T. aestivum*.cv Rosella (hexaploid)

          The horizontal axis indicates the size of the product in base pairs, the vertical axis shows arbitrary fluorescence units. The various arrows indicate the products

35   of different genomes: A, A genome, B, B genome, D, D genome, U, unassigned additional product.

Figure 26 shows the results obtained by amplification using the SBE II-Intron 10 primer set (primer set i1: da5.seq and WBE2E11R on the wheat lines:

    (i) *T. aestivum* cv. Chinese Spring ditelosomic line 2AS.

    (ii) *T. aestivum* Chinese Spring nullisomic/tetrasomic line N2BT2A.

    (iii) *T. aestivum* Chinese Spring nullisomic/tetrasomic line N2DT2B.

The horizontal axis indicates the size of the product in base pairs, the vertical axis shows arbitrary fluorescence units. The various arrows indicate the products of different genomes: A, A genome, B, B genome, D, D genome.

Figure 27 shows the results of transient expression assays typical of each promoter and target tissue. The photographs (40 x magnification) of representative tissue resulting from the transient expression assays typical of each promoter and target tissue revealed under a Leica microscope with blue light illumination. Photographs were taken 48 to 72 hours after tissue bombardment. The promoter constructs are listed as follows, (with the panels showing endosperm, embryo and leaf expression listed in respective order): pact_jsgfp_nos (panels a,g and m); pwsssIpro1gfpNOT (panels b, h and n); pwsssIpro2gfpNOT (panels c, i and o); psbeIIpro1gfpNOT (panels d, j and p); psbeIIpro2gfpNOT (panels e, k and q); pZLgfpNOT (Panels f, l and r).

Example 1   Identification of Gene Encoding SBE I

Construction of Genomic Library and Isolation of Clones

The genomic library used in this study was constructed from *Triticum tauschii*, var. strangulata, accession number CPI 100799. Of all the accessions of *T. tauschii* surveyed, the genome of CPI 100799 is the most closely related to the D genome of hexaploid wheat.

Triticum tauschii, var strangulata (CPI accession number 110799) was kindly provided by Dr E Lagudah. Leaves were isolated from plants grown in the glasshouse.

DNA was extracted from leaves of *Triticum tauschii*
5 using published methods (Lagudah *et al*, 1991), partially digested with *Sau*3A, size fractionated and ligated to the arms of lambda GEM 12 (Promega). The ligated products were used to transfect the methylation-tolerant strain PMC 103 (Doherty *et al.* 1992). A total of 2×10^6 primary plaques
10 were obtained with an average insert size of about 15 kb. Thus the library contains approximately 6 genomes worth of *T. tauschii* DNA. The library was amplified and stored at 4°C until required.

Positive plaques in the genomic library were
15 selected as those hybridising with the 5' end of a maize starch branching enzyme I cDNA (Baba *et al*, 1991) using moderately stringent conditions as described in Rahman *et al*, (1997).

20 **Preparation of Total RNA from Wheat**

Total RNA was isolated from leaves, pre-anthesis pericarp and different developmental stages of wheat endosperm of the cultivar, Hartog and Rosella. This material was collected from both the glasshouse and the
25 field. The method used for RNA isolation was essentially the same as that described by Higgins *et al* (1976). RNA was then quantified by UV absorption and by separation in 1.4% agarose-formaldehyde gels which were then visualized under UV light after staining with ethidium bromide
30 (Sambrook *et al*, 1989).

DNA and RNA analysis

DNA was isolated and analysed using established protocols (Sambrook *et al*, 1989). DNA was extracted from
35 wheat (cv. Chinese Spring) using published methods (Lagudah *et al*, 1991). Southern analysis was performed essentially as described by Jolly *et al* (1996). Briefly, 20 μg wheat

- 20 -

DNA was digested, electrophoresed and transferred to a nylon membrane. Hybridisation was conducted at 42°C in 25% or 50% formamide, 2 x SSC, 6% Dextran Sulphate for 16h and the membrane was washed at 60°C in 2 x SSC for 3 x 1h unless

5 otherwise indicated. Hybridisation was detected by autoradiography using Fuji X-Omat film.

RNA analysis was performed as follows. 10 µg of total RNA was separated in a 1.4% agarose-formaldehyde gel and transferred to a nylon Hybond N$^+$ membrane (Sambrook et

10 al, 1989), and hybridized with cDNA probe at 42°C in Khandjian hybridizing buffer (Khandjian, 1989). The 3' part of wheat SBE I cDNA (designated wSBE I-D43, see Table 1) was labelled with the Rapid Multiprime DNA Probe Labelling Kit (Amersham) and used as probe. After washing at 60°C with

15 2 x SSC, 0.1% SDS three times, each time for about 1 to 2 hours, the membrane was visualized by overnight exposure at -80°C with X-ray film, Kodak MR.

Example 2   Frequency of Recovery of SBE I Type Clones

20   from the Genomic Library

An estimated 2 x 10^6 plaques from the amplified library were screened using an EcoRI fragment that contained 1200 bp at the 5' end of maize SBE I (Baba et al, 1991) and twelve independent isolates were recovered and purified.

25 This corresponds to the screening of somewhat fewer than the 2 x 10^6 primary plaques that exist in the original library (each of which has an average insert size of 15 kb) (Maniatis et al, 1982), because the amplification may lead to the representation of some sequences more than others.

30 Assuming that the amplified library contains approximately three genomes of T. tauschii, the frequency with which SBE I-positive clones were recovered suggests the existence of about 5 copies of SBE I type genes within the T. tauschii genome.

35   Digestion of DNA from the twelve independent isolates by the restriction endonuclease BamHI followed by hybridisation with a maize SBE I clone, suggested that the

genomic clones could be separated into two broad classes
(Figure 1). One class had 10 members and a representative
from this class is the clone λE1 (Figure 1, lane 1); λE6
(Figure 1, lane 3) is a member of this class, but is missing
5   the 5' end of the E1-SBE I gene because the SBE I gene is at
the extremity of the cloned DNA. Further hybridisation
studies at high stringency with the extreme 5' and
3' regions of the SBE I gene contained in λE1 suggested that
the other clones contained either identical or very closely
10 related genes.

        The second family had two members, and of these
clone λE7 (Figure 1, lane 4) was arbitrarily selected for
further study. These two members did not hybridise to
probes from the extreme 5' and 3' regions of the SBE I gene
15 that were contained in λE1, indicating that they were a
distinct sub-class.

        The DNA from *T. tauschii* and the lambda clones λE1
and λE7 was digested with *Bam*HI and hybridised with
fragment E1.1, as shown in Figure 2. This fragment contains
20 sequences that are highly conserved (85% sequence identity
over 0.3 kB between λE1 and λE7), corresponding to exons 3,
4 and 5 of the rice gene. The bands in the genomic DNA at
0.8 kb and 1.0 kb correspond to identical sized fragments
from λE1 and λE7, as shown in Figure 2; these are
25 fragments E1.1 and E7.8 of λE1 and λE7 genomic clones
respectively. Thus the arrangement of genes in the genomic
clones is unlikely to be an artefact of the cloning
procedure. There are also bands in the genomic DNA of
approximately 2.5 kb, 4.8 kb and 8 kb in size which are not
30 found from the digestion of λE1 or λE7; these could
represent genes such as the 5' sequences of wSBE I-D1 or
wSBE I-D3; see below.

        Example 3   Tandem Arrangement of SBE I Type Genes in
35   the *T. tauschii* Genome
        Basic restriction endonuclease maps for λE1 and
λE7 are shown in Figure 3. The map was constructed by

performing a series of hybridisations of *Eco*RI or *Bam*HI
digested DNA from λE1 or λE7. The probes used were the
fragments generated from *Bam*HI digestion of the relevant
clone. Confirmation of the maps was obtained by PCR

5   analysis, using primers both within the insert and also from
the arms of lambda itself. PCR was performed in 10 µl
volume using reagents supplied by Perkin-Elmer. The primers
were used at a concentration of 20 µM. The program used was
94°C, 2 min, 1 cycle, then 94°C, 30 sec; 55°C, 30 sec; 72°C,

10 1min for 36 cycles and then 72°C, 5 min; 25°C, 1 min.

Sequencing was performed on an ABI sequencer using
the manufacturer's recommended protocols for both dye primer
and dye terminator technologies. Deletions were carried out
using the Erase-a-base kit from Promega.

15 Sequence analysis was carried out using the GCG
version 7 package of computer programs (Devereaux *et al*,
1984).

The PCR products were also used as hybridisation
probes. The positioning of the genes was derived from

20 sequencing the ends of the *Bam*HI subclones and also from
sequencing PCR products generated from primers based on the
insert and the lambda arms. The results indicate that there
is only a single copy of a SBE I type gene within λE1.
However, it is clear that λE7 resulted from the cloning of a

25 DNA fragment from within a tandem array of the SBE I type
genes. Of the three genes in the clone, which are named as
wSBE I-D1, wSBE I-D2 and wSBE I-D3); only the central one
(wSBE I-D2) is complete.

30 Example 4   Construction and Screening of cDNA Library
A wheat cDNA library was constructed from the
cultivar Rosella using pooled RNA from endosperm at 8, 12,
18 and 20 days after anthesis.

The cDNA library was prepared from poly A$^+$ RNA

35 that was extracted from developing wheat grains (cv.
Rosella, a hexaploid soft wheat cultivar) at 8, 12, 15, 18,
21 and 30 days after anthesis. The RNA was pooled and used

to synthesise cDNA that was propagated in lambda ZapII
(Stratagene).

The library was screened with a genomic fragment
from λE7 encompassing exons 3, 4 and 5 (fragment E7.8 in
5   Figure 3). A number of clones were isolated. Of these an
apparently full-length clone appeared to encode an unusual
type of cDNA for SBE I. This cDNA has been termed SBE I-D2
type cDNA. The putative protein product is compared with
the maize SBE I and rice SBE I type deduced amino acid
10   sequences in Figure 4. The main difference is that this
putative protein product is shorter at the C-terminal end,
with an estimated molecular size of approximately 74 kD
compared with 85 kDa for rice SBE I (Kawasaki *et al*, 1993).
Note that amino acids corresponding to exon 9 of rice are
15   missing in SBE I-D2 type cDNA, but those corresponding to
exon 10 are present. There are no amino acid residues
corresponding to exons 11-14 of rice; furthermore, the
sequence corresponding to the last 57 amino acids of
SBE I-D2 type has no significant homology to the sequence of
20   the rice gene.

We expressed SBE I-D2 type cDNA in *E. coli* in
order to examine its function. The cDNA was expressed as a
fusion protein with 22 N-terminal residues of β-galacto-
sidase and two threonine residues followed by the SBE I-D2
25   cDNA sequence either in or out of frame. Although an
expected product of about 75 kDa in size was produced from
only the in-frame fusion, we could not detect any enzyme
activity from crude extracts of *E. coli* protein.
Furthermore the in-frame construct could not complement an
30   *E. coli* strain with a defined deletion in glycogen
branching, although other putative branching enzyme cDNAs
have been shown to be functional by this assay (data not
shown). It is therefore unclear whether the wSBE I-D2 gene
in λE7 codes for an active enzyme *in vivo*.
35

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<u>Example 5</u>   <u>Gene Structure in E7</u>

i. Sequence of wSBE I-D2

　　　　We sequenced 9.2 kb of DNA that contained
wSBE I-D2. This corresponds to fragments 7.31, 7.8 and
5   7.18. Fragment 7.31 was sequenced in its entirety (4.1 kb),
but the sequence of about 30 bases about 2 kb upstream of
the start of the gene could not be obtained because it was
composed entirely of Gs. Elevation of the temperature of
sequencing did not overcome this problem. Fragments 7.8
10   (1 kb) and 7.18 (4 kb) were completely sequenced, and
corresponded to 2 kb downstream of the last exon detected
for this gene. It was clear that we had isolated a gene
which was closely related (approximately 95% sequence
identity) to the SBE I-D2 type cDNA referred to above,
15   except that the last 200 bp at the 3' end of the cDNA are
not present. The wSBE I-D2 gene includes sequences
corresponding to rice exon 11 which are not in the cDNA
clone. In addition it does not have exons 9, 12, 13 or 14;
these are also absent from the SBE I-D2 type cDNA. The
20   first two exons show lower identity to the corresponding
exons from rice (approximately 60%) (Kawasaki *et al*, 1993)
than to the other exons (about 80%). A diagrammatic exon-
intron structure of the wSBE I-D2 gene is indicated in
Figure 5. The restriction map was confirmed by sequencing
25   the PCR products that spanned fragments 7.18 and 7.8 and 7.8
and E7.31 (see Figure 3) respectively.

ii. Sequence of wSBE I-D3

　　　　This gene was not sequenced in detail, as the
30   genomic clone did not extend far enough to include the 5'
end of the sequence. The sequence is of a SBE-I type. The
orientation of the gene is evident from sequencing of the
relevant *Bam*HI fragments, and was confirmed by sequence
analysis of a PCR product generated using primers from the
35   right arm of lambda and a primer from the middle of the
gene. The sequence homology with wSBEI-D2 is about 80% over
the regions examined. The 2 kb sequenced corresponded to

exons 5 and 6 of the rice gene; these sequences were
obtained by sequencing the ends of fragments 7.5, 7.4 and
7.14 respectively, although the sequences from the left end
of fragment 7.14 did not show any homology to the rice
5   sequences. The gene does not appear to share the 3' end of
SBE I-D2 type cDNA, as a probe from 500 bp at the 3' end of
the cDNA (including sequences corresponding to exons 8 and
10 from rice) did not hybridise to fragment 7.14, although
it hybridised to fragment 7.18.
10

iii.   Sequence of wSBE I-D1
         This gene was also not sequenced in detail, as it
was clear that the genomic clone did not extend far enough
to include the 5' sequences. Limited sequencing suggests
15   that it is also a SBE I type gene. The orientation relative
to the left arm of lambda was confirmed by sequencing a PCR
product that used a primer from the left arm of lambda and
one from the middle of the gene (as above). Its sequence
homology with wSBE I-D2 ,D3 and D4 (see below) is about 75%
20   in the region sequenced corresponding to a part of exon 4 of
the rice gene.
         Starch branching enzymes are members of the α-
amylase protein family, and in a recent survey Svensson
(1994) identified eight residues in this family that are
25   invariant, seven in the catalytic site and a glycine in a
short turn. Of the seven catalytic residues, four are
changed in SBE I-D2 type. However, additional variation in
the 'conserved' residues may come to light when more plant
cDNAs for branching enzyme I are available for analysis. In
30   addition, although exons 9, 11, 12, 13 and 14 from rice are
not present in the SBE I-D2 type cDNA, comparison of the
maize and rice SBE I sequences indicate that the 3' region
(from amino acid residue 730 of maize) is much more variable
than the 5' and central regions. The active sites of rice
35   and maize SBE I sequences, as indicated by Svensson (1994),
are encoded by sequences that are in the central portion of
the gene. When SBE II sequences from *Arabidopsis* were

compared by Fisher *et al* (1996) they also found variation at the 3' and 5' ends. SBE I-D2 type cDNA may encode a novel type of branching enzyme whose activity is not adequately detected in the current assays for detecting branching

5 enzyme activity; alternatively the cDNA may correspond to an endosperm mRNA that does not produce a functional protein.

Example 6   Cloning of the cDNA corresponding to the wSBE I-D4 gene

10   The first strand cDNAs were synthesized from 1 µg of total RNA, derived from endosperm 12 days after pollination, as described by Sambrook *et al* (1989), and then used as templates to amplify two specific cDNA regions of wheat SBE I by PCR.

15   Two pairs of primers were used to obtain the cDNA clones BED1 and BED3 (Table 1). Primers used for cloning of BED3 were the degenerate primer NTS5'

5' GGC NAC NGC NGA G/AGA C/TGG 3'   (SEQ ID NO.1),
20

        based on the N-terminal sequence of the purified wheat endosperm SBE I protein, in which the 5' end of the primer is at position 168 of wSBE I-D4 cDNA, as shown in Table 1, based on the N-terminal sequence of wheat SBE I,

25 and the primer NTS3'.

5' TAC ATT TCC TTG TCC ATCA 3'   (SEQ ID NO.2)

        in which the 5' end is at position 1590 of

30 wSBE I-D4 cDNA, (see Table 1), designed to anneal to the conserved regions of the nucleotide sequences of BED5 and the maize and rice SBE I cDNAs. For clone BED1, the primers used were BEC5'

35 5' ATC ACG AGA GCT TGC TCA   (SEQ ID NO.3)

in which the 5' end is at position 1 of wSBE I-D4
cDNA (see Table 1); the sequence was based on the wSBE I-D4
gene, and BEC3'

5   5' CGG TAC ACA GTT GCG TCA TTT TC 3'   (SEQ ID NO.4)

in which the 5' end is at position 334 of
wSBE I-D4 cDNA (see Table 1), and the sequence was based on
BED 3.

10

Example 7   Identification of the gene from the _Triticum_
                   tauschii SBE I family which is expressed in
                   the endosperm

We have isolated two classes of SBE I genomic
15   clones from _T. tauschii_. One class contained two genomic
clone isolates, and this class has been characterised in
some detail (Rahman _et al_, 1997). The complete gene
contained within this class of clones was termed _wSBE I-D2_;
there were additional genes at either ends of the clone, and
20   these were designated _wSBE I-D1_ and _wSBE I-D3_. The other
class contained nine genomic clone isolates. Of these λE1
was arbitrarily taken as a representative clone, and its
restriction map is shown in Figure 3; the SBE I gene
contained in this clone was called _wSBE I-D4_.
25   Fragments E1.1 (0.8 kb) and E1.2 (2.1 kb) and
fragments E1.7 (4.8 kb) and E1.5 (3 kb) respectively were
completely sequenced. Fragment E1.7 was found to encode the
N-terminal of the SBE I, which is found in the endosperm as
described in Morell _et al_ (1997). This is shown in
30   Figure 6. Using antibodies raised against the N-terminal
sequence, Morell _et al_ (1997) found that the D genome
isoform was the most highly expressed in the cultivars
Rosella and Chinese Spring. We have thus isolated from
T. tauschii a gene, _wSBE I-D4_, whose homologue in the
35   hexaploid wheat genome encodes the major isoform for SBE I
that is found in the wheat endosperm.

Table 1
Location of structural features and probes within wSBE I-D4 sequence.

5   A. Location of exons by comparison with the cDNA sequence of Repellin et al., (1997). Accession number Y12320.

Exon number	Start posn	End posn
1	4890	4987
2	5082	5149
3	5524	5731
4	5819	5888
5	6149	6318
6	6519	7424
7	7744	7860
8	8015	8077
9	8562	8670
10	9137	9237
11	9421	9488
12	9580	9661
13	9781	9897
14	9990	10480

25   B.   Other features.

Name of feature.	wSBE I-D4. sequence	D4 cDNA sequence.
Putative initiation of translation	4900	11
Mature N-terminal sequence of SBE I	5550	124
End of translated SBE I sequence	10225	2431
End of D4 cDNA sequence	10461	2687
wSBE I-D45	4870,5860	1,354
wSBE I-D43	10116,10435	2338,2657
E1.1	5680,6400	380,630
BED 1		1,354
BED 2		169,418
BED 3		151,1601
BED 4		867,2372
BED 5		867,2687
Endosperm box like motif TGAAAAGT	4480,590	
CAAAT motif	4863	
TATAAA motif	4833	

All nine genomic clones of the λE1 type isolated
from *T. tauschii* appear to contain the *wSBE I-D4* gene, or
very similar genes, on the basis of PCR amplification and
hybridisation experiments. However, the restriction
5   patterns obtained for the clones differ with *Bam*HI and
*Eco*RI, among other enzymes, indicating that either the
clones represent near-identical but distinct genes or they
represent the same gene isolated in distinct products of the
*Sau*3A digest used to generate the library.
10

Example 8   Investigation of other SBE I genomic clones
                 isolated
        All ten members of the λE1-like class of SBE I
genomic clones were investigated by hybridisation with
15   probes derived from fragment E1.7 (sequence *wSBE I-D45*,
encoding the translation start signal and the first
100 amino acids from the N-terminal end and intron
sequences; see Table 1) and from fragment E1.5 (sequence
wSBE I-D43, corresponding largely to the 3' untranslated
20   sequence and containing intron sequences, see Table 1). The
results obtained were consistent with one type of gene being
isolated in different fragments in the different clones, as
shown in Figure 7. The PCR products were obtained from the
clones λE1, 2, 9, 14, 27, 31 and 52. These hybridised to
25   *wSBE I-D45* using primers that amplify near the 5' end of the
gene (positions 5590-6162 of *wSBE I-D4*). Sequencing showed
no differences in sequence of a 200 bp product.
        Analysis of the promoter for *wSBE I-D4* allows us
to investigate the presence of motifs previously described
30   for promoters that regulate gene expression in the
endosperm. Forde *et al* (1985) compared prolamin promoters,
and suggested that the presence of a motif approximately
-300 bp upstream of the transcription start point, called
the endosperm box, was responsible for endosperm-specific
35   expression. The endosperm box was subsequently considered
to consist of two different motifs: the endosperm motif (EM)
(canonical sequence TGTAAAG) and the GCN 4 motif (canonical

sequence G/ATGAG/CTCAT). The GCN4 box is considered to
regulate expression according to nitrogen availability
(Muller and Knudsen, 1993). The *wSBE I-D4* promoter contains
a number of imperfect EM-like motifs at approximately -100,
5   -300 and -400 as well as further upstream. However, no GCN4
motifs could be found, which lends support to the idea that
this motif regulates response to nitrogen, as starch
biosynthesis is not as directly dependent on the nitrogen
status of the plant as storage protein synthesis. Comparison
10 of the promoters for *wSBE I-D4* and *D2* (Rahman *et al*, 1997)
indicates that although there are no extensive sequence
homologies there is a region of about 100 bp immediately
before the first encoded methionine where the homology is
61% between the two promoters. In particular there is an
15 almost perfect match in the sequence over twenty base pairs
CTCGTTGCTTCC/TACTCCACT, (positions 4723-4742 of the *wSBE I*
sequence), but the significance of this is hard to gauge, as
it does not occur in the rice promoter for SBE I. The
availability of more promoters for starch biosynthetic
20 enzymes may allow firmer conclusions to be drawn. There are
putative CAAT and TATA motifs at positions 4870 and 4830
respectively of *wSBE I-D4* sequence. The putative start of
translation of the mRNA is at position 4900 of *wSBE I-D4*.
        Figure 5 shows the structure of the *wSBE I-D4*
25 gene, compared with the genes from rice and wheat (Kawasaki
et al, 1993; Rahman *et al*, 1997). The rice *SBE I* has 14
exons compared with 13 for *wSBE I-D4* and 10 for *wSBE I-D2*.
There is good conservation of exon-intron structure between
the three genes, except at the extreme 5' end. In particular
30 the sizes of intron 1 and intron 2 are very different
between rice *SBE I* and *wSBE I-D4*.

Example 9   Isolation of cDNA for SBE I
        Using the maize starch branching enzyme I cDNA as
35 a probe (Baba *et al*, 1991), 10 positive plaques were
recovered by screening approximately 10^5 plaques from a
wheat endosperm cDNA library prepared from the cultivar

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Rosella, as described in Example 4. On purifying and sequencing these plaques it was clear that even the longest clone (BED5, 1822 bp) did not encode the N-terminal sequence obtained from protein analysis. Degenerate primers based on

5   the wheat endosperm SBE I protein N-terminal sequence (Morell *et al*, 1997) and the sequence from BED5 were then used to amplify the 5' region: this produced a cDNA clone termed BED 3 (Table 1 and Figure 8). This cDNA clone overlapped extensively and had 100% sequence identity with

10   BED5 and BED4 (Figure 8). As almost the entire protein N-terminal sequence had been included in the primer sequence design, this did not provide independent evidence of the selection of a cDNA sequence in the endosperm that encoded the protein sequence of the main form of SBE I. Using a

15   BED3 to screen a second cDNA library produced BED2, which is shorter than BED3 but confirmed the BED3 sequence at 100% identity between positions 169 and 418 (Figure 8 and Table 1). In addition the entire cDNA sequence for BED3 could be detected at a 100% match in the genomic clone λE1.

20   Primers based on the putative transcription start point combined with a primer based on the incomplete cDNAs recovered were then used to obtain a PCR product from total endosperm RNA by reverse transcription. This led to the isolation of the cDNA clone, BED1, of 300 bp, whose location

25   is shown in Figure 8. By analysing this product, a sequence was again obtained that could be found exactly in the genomic clone λE1, and which overlapped precisely with BED3.

The N-terminal of the protein matches that of SBE I isolated from wheat endosperm by Morell *et al* (1997),

30   and thus the *wSBE I-D4* cDNA represents the gene for the predominant SBE I isoform expressed in the endosperm. The encoded protein is 87 kDa; this is similar to proteins encoded by maize (Baba *et al*, 1991) and rice (Nakamura *et al*, 1992) cDNAs for SBE I and is distinct from the *wSBE I-D2*

35   cDNA described previously, in which the encoded protein was 74 kDa (Rahman *et al*, 1997).

Five cDNA clones were sequenced and their
sequences were assembled into one contiguous sequence using
a GCG program (Devereaux *et al*, 1984). The arrangement of
these sequences is illustrated in Figure 8, the nucleotide
5   sequence is shown in SEQ ID No:5, and the deduced amino acid
sequence is shown in SEQ ID No:6. The intact cDNA sequence,
wSBE I-D4 cDNA, is 2687 bp and contains one large open
reading frame (ORF), which starts at nucleotides 11 to 13
and ends at nucleotides 2432 to 2434. It encodes a
10 polypeptide of 807 amino acids with a molecular weight of
87 kDa. Comparison of the amino acid sequence encoded by
wSBE I-D4 cDNA with that encoded by maize and rice *SBE I*
cDNAs showed that there is 75-80% identity between any of
two these sequences at the nucleotide level and almost 90%
15 at the amino acid level. Alignment of these three
polypeptide sequences, as shown in Figure 4, along with the
deduced sequences for pea, potato and *wSBE I-D2* type cDNA,
indicated that the sequences in the central region are
highly conserved, and sequences at the 5' end (about
20 80 amino acids) and the 3' end (about 60 amino acids) are
variable.

Svensson *et al* (1994) indicated that there were
several invariant residues in sequences of the α-amylase
super-family of proteins to which SBE I belongs. In the
25 sequence of maize SBE I these are in motifs commencing at
amino acid residue positions 341, 415, 472, 537
respectively; these are also encoded in the *wSBE I-D4*
sequence (SEQ ID No:9), further supporting the view that
this gene encodes a functional enzyme. This is in contrast
30 to the results with the *wSBE I-D2* gene, where three of the
conserved motifs appear not to be encoded (Rahman *et al*,
1997).

There is about 90% sequence identity in the
deduced amino acid sequence between *wSBE I-D4* cDNA and rice
35 *SBE I* cDNA in the central portion of the molecule (between
residues 160 and 740 for the deduced amino acid product from
wSBE I-D4 cDNA). The sequence identity of the deduced amino

acid sequence of the *wSBE I-D4* cDNA to the deduced amino
acid sequence of *wSBE I-D2* is somewhat lower (85% for the
most conserved region, between residues 285 to 390 for the
deduced product of *wSBE I-D4* cDNA). Surprisingly, however,

5   *wSBE I-D4* cDNA is missing the sequence that encodes amino
acids at positions 30 to 58 in rice SBE I (see Figure 4).
This corresponds to residues within the transit peptide of
rice SBE I. A corresponding sequence also occurs in the
deduced amino acid sequence from maize *SBE I* (Baba *et al*,

10 1991) and *wSBE I-D2* type cDNA (Rahman *et al*, 1997).
Consequently the transit sequence encoded by *wSBE I-D4* cDNA
is unusally short, containing only 38 amino acids, compared
with 55-60 amino acids deduced for most starch biosynthetic
enzymes in cereals (see for example Ainsworth, 1993; Nair *et*

15 *al*, 1997). The *wSBE I-D4* gene does contain this sequence,
but this does not appear to be transcribed into the major
species of RNA from this gene, although it can be detected
at low relative abundance. This raises the possibility of
alternative splicing of the *wSBE I-D4* transcript, and also

20 the question of the relative efficiency of
translation/transport of the two isoforms. The possibility
of alternative splicing in both rice and wheat has been
considered for soluble starch synthase (Baba *et al*,1993
Rahman *et al*, 1995). Alternative splicing of soluble starch

25 synthase would give a transit sequence of 40 amino acids,
which is the same length proposed for the product of
wSBE I-D4 cDNA.

We have previously used probes based on exons 4, 5
and 6 (E7.8 and E1.1, see Rahman *et al.*, 1997) of *wSBE-D2* to

30 probe wheat and *T. tauschii* genomic DNA cleaved with *Pvu*II
and *Bam*HI respectively. This region is highly conserved
within rice *SBE I*, *wSBE I-D2* and *wSBE I-D4* and produced ten
bands with wheat DNA and five with *T. tauschii* DNA. Neither
*Pvu*II nor *Bam*HI cleaved within the probe sequences,

35 suggesting that each band represented a single type of SBE I
gene. We have described four SBE I genes from *T. tauschii*:
wSBE I-D1, *wSBE I-D2*, *wSBE I-D3* and *wSBE I-D4* (Rahman *et al*,

1997 and this specification), and so we may have accounted
for most of the genes in *T. tauschii* and, by extension, the
genes from the D genome of wheat. In wheat, at least two
hybridising bands could be assigned to each of
5   chromosomes 7A, 7B and 7D.

Example 10   Tissue specificity and expression during
                  endosperm development

The 300 bp of 3' untranslated sequence of
10 *wSBE I-D4* cDNA does not show any homology with either the
wSBE I-D2 type cDNA that we have described earlier (Rahman
et al, 1997) or with BE-I from rice, as shown in Figure 5.
We have called this sequence *wSBE I-D43C* (see SEQ ID No:9).
It seemed likely that *wSBE I-D43C* would be a specific probe
15 for this class of SBE-I, and thus it was used to investigate
the tissue specificity. Hybridization of RNA from endosperm
of hexaploid *T. tauschii* cultures with SBE I, SBE II, SSS I,
DBE I, wheat actin, and wheat ribosomal RNA was examined.
RNA was purified at various numbers of days after anthesis
20 from plants grown with a 16 h photoperiod at 13 °C (night)
and 18 °C (day). The age of the endosperms from which RNA
was extracted in days after anthesis is given above the
lanes in the blot. Equivalent amounts of RNA were
electrophoresed in each lane. The probes used are identified
25 in Tables 1 and 2.

The results are shown in Figures 9a to 9g. An RNA
species of about 2700 bases in size was found to hybridise.
This is very close to the size of the *wSBE I-D4* cDNA
sequence. RNA hybridising to wSBE-I-D43C is most abundant
30 at the mid-stage of endosperm development, as shown in
Figure 9a, and in field grown material is relatively
constant during the period 12-18 days, the time at which
there is rapid starch and storage protein accummulation
(Morell *et al*, 1995).

35   The sequence contained within the *wSBE I-D4* gene
appears to be expressed only in the endosperm (Figure 9a,
Figure 9b). We could not detect any expression in the leaf.

This could be because another isoform is expressed in the
leaf, and/or because the amount of SBE I present in the leaf
is much less than what is required in the endosperm.
Isolation of SBE I clones from a leaf cDNA library would
5   enable this question to be resolved.

Example 11   Intron-Exon Structure of SBE I

By comparison of the cDNA sequence of SBE I
(Repellin et al, 1997) with that of wSBE I-D4 we can deduce
10   the intron-exon structure of the gene for the major isoform
of SBE I that is found in the endosperm. The structure
contains 14 exons compared to 14 for rice (Kawasaki et al,
1993). These 14 exons are spread over 6 kb of sequence, a
distance similar to that found in both rice SBE I and
15   wSBE I-D2. A dotplot comparison of wSBE I-D4 sequence and
that of rice SBE I sequence, depicted in Figure 10, shows
good sequence identity over almost the entire gene starting
from about position 5100 of wSBE I-D4; the identity is poor
over the first 5 kb of sequence corresponding largely to the
20   promoter sequences. The sequence identity over introns
(about 60%) is lower than over exons (about 85%).

Example 12   Repeated Sequences in SBE I

Sequencing of wSBE I-D4 revealed there was a
25   repeated sequence of at least 300 bp contained in a 2kb
fragment about 600 bp after the 3' end of the gene. We have
called this sequence wSBE I-D4R (SEQ ID NO: 9). This
repeated sequence is within fragment E1.5 (Figure 3 and
Table 1) and is flanked by non-repetitive sequences from the
30   genomic clone. We have previously shown that the
restriction pattern obtained by digesting λE1 with the
restriction enzyme BamHI is also obtained when T. tauschii
DNA is digested. Thus wSBE I-D4R is unlikely to be a
cloning artefact. A search of the GenBank Database revealed
35   that wSBE I-D4R shared no significant homology with any
sequence in the database. Hybridisation experiments with
wSBE I-D4R showed that all of the other SBE I-D4 type

genomic clones (except number 29) contained this repeated
sequence (data not shown). The *wSBE I-D4R* sequence was not
highly repeated and occurred in the wheat genome with a
similar frequency as the *wSBE I-D4* sequence.

5   When *SBE I-D4R* was used as the probe on wheat DNA
from the nulli-tetra lines, four bands were obtained; two of
these bands could be assigned to chromosome 7A and the
others to chromosomes 7B and 7D (Figure 11). One of the two
*Bam*HI fragments from wheat DNA which could be assigned to

10   chromosome 7A was distinct from the single band from
chromosome 7A detected using *wSBE I-D43* as the probe; the
other three bands coincided in the autoradiograph with bands
obtained with *wSBE I-D43*, and are likely to represent the
same fragment. However, one of these fragments was distinct

15   from the *Bam*HI fragment that hybridised to the *wSBE I-D43*
sequence. In *wSBE I-D4* (see SEQ ID No:9), the *wSBE I-D43*
sequence is only 300 bp upstream of *wSBE I-D4R*, and occurs
in the same *Bam*HI fragment. These results suggest that the
wSBE I-D4R sequence can occur independently of *wSBE I-D4* in

20   the wheat genome.

Example 13   Isolation of Genomic Clones Encoding SBE II
          Screening of a cDNA library, prepared from the
wheat endosperm as described in Example 4, with the maize

25   BE I clone (Baba *et al*, 1991) at low stringency led to the
isolation of two classes of positive plaques. One class was
strongly hybridising, and led to the isolation of wheat
SBE I-D2 type and SBE I-D4 type cDNA clones, as described in
Example 5 and in Rahman *et al* (1997). The second class was

30   weakly hybridising, and one member of this class was
purified. This weakly hybridising clone was termed SBE-9,
and on sequencing was found to contain a sequence that was
distinct from that for SBE I. This sequence showed greatest
homology to maize BE II sequences, and was considered to

35   encode part of the wheat SBE II sequence.
          The screening of approximately 5 x 10^5 plaques
from a genomic library constructed from *T. tauschii* (see

Example 1) with the SBE-9 sequence led to the isolation of
four plaques that were positive. These were designated
wSBE II-D1 to wSBE II-D4 respectively, and were purified and
analysed by restriction mapping. Although they all had
5   different hybridization patterns with SBE-9, as shown in
Figure 12, the results were consistent with the isolation of
the same gene in different-sized fragments.

Example 14   Identification of the N-terminal sequence of
10   SBE II
         Sequencing of the SBE II gene contained in
clone 2, termed SBE II-D1 (see SEQ ID No:10), showed that it
coded for the N-terminal sequence of the major isoform of
SBE II expressed in the wheat endosperm, as identified by
15   Morell et al (1997). This is shown in Figure 13.

Example 15   Intron-Exon Structure of the SBE II Gene
         In addition to encoding the N-terminal sequence of
sBE II, as shown in Example 10, the cDNA sequence reported
20   by Nair et al (1997) was also found to have 100% sequence
identity with part of the sequence of wSBE II-D1. Thus the
intron-exon structure can be deduced, and this is shown in
Figure 14. The positions of exons and other major structural
features of the SBE II gene are summarized in Table 2.
25

Example 16   Number of SBE II Genes in T. tauschii and
                 Wheat
         Hybridisation of the SBE II conserved region with
T. tauschii DNA revealed the presence of three gene classes.
30   However, in our screening we only recovered one class.
Hybridisation to wheat DNA indicated that the locus for
SBE II was on chromosome 2, with approximately 5 loci in
wheat; most of these appear to be on chromosome 2D, as shown
in Figure 15.
35

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Table 2
Positions of structural features in wSBE II-D1.

A. Positions of exons.

Exon number	Genomic start	Genomic finish
1	1058	1336
2	1664	1761
3	2038	2279
4	2681	2779
5	2949	2997
6	3145	3204
7	3540	3620
8	3704	3825
9	4110	4188
10	4818	4939
11	5115	5234
12	6209	6338
13	6427	6549
14	6739	6867
15	7447	7550
16	8392	8536
17	9556	9703
18	9839	9943
19	10120	10193
20	10395	10550
21	10928	11002
22	11092	11475

B.   Other structural features within the wSBE II-D1 DNA
      sequence

Putative initiation of translation	1214
Mature N-terminal sequence of SBE II.	1681
wSBE II-D13	11116 to 11448
Endosperm box like motif TGAAAAGT	521
Endosperm box like motif TGAAAGT	565
Endpsperm box like motif CGAAAAT	669
Endosperm box like motif TAAATGT	768
CAAAAT motif	784
TCAATT motif	1108
TATAAA motif	799
AATTAA motif	1110

<u>Example 17</u>   <u>Expression of SBE II</u>

Investigation of the pattern of expression of
SBE II revealed that the gene was only expressed in the
endosperm. However the timing of expression was quite
5   distinct from that of SBE I, as illustrated in
Figures 9a, 9b and 9c.

SBE I gene expression is only clearly detectable
from the mid-stage of endosperm development (10 days after
anthesis in Figure 9b), whereas SBE II gene expression is
10   clearly seen much earlier, in endosperm tissue at 5-8 days
after development (Figures 9a and 9c), corresponding to an
early stage of endosperm development. The hybridisation of
wheat endosperm mRNA with the actin and ribosomal RNA genes
is shown as controls (Figures 9fa and 9g, respectively).
15

<u>Example 18</u>   <u>Cloning of Wheat Soluble Starch Synthase</u>
                <u>cDNA</u>

A conserved sequence region was used for the
synthesis of primers for amplification of SSS I by
20   comparison with the nucleotide sequences encoding soluble
starch synthases of rice and pea. A 300 bp RT-PCR product
was obtained by amplification of cDNA from wheat endosperm
at 12 days post anthesis. The 300 bp RT-PCT product was
then cloned, and its sequence analysed. The comparison of
25   its sequence with rice SSS cDNA showed about 80% sequence
homology. The 300 bp RT-PCR product was 100% homologous to
the partial sequence of a wheat SSS I in the database
produced by Block *et al* (1997).

The 300 bp cDNA fragment of wheat soluble starch
30   synthase thus isolated was used as a probe for the screening
of a wheat endosperm cDNA library (Rahman *et al*, 1997).
Eight cDNA clones were selected. One of the largest cDNA
clones (sm2) was used for DNA sequencing analysis, and gave
a 2662 bp nucleotide sequence, which is shown in SEQ ID
35   NO:14. A large open reading frame of this cDNA encoded a
647 amino acid polypeptide, starting at nucleotides 247 to
250 and terminating at nucleotides 2198 to 2200. The

deduced polypeptide was shown by protein sequence analysis
to contain the N-terminal sequence of a 75 kDa granule-bound
protein (Rahman et al, 1995). This is illustrated in
Figure 16. The location of the 75 kDa protein was

5   determined for both the soluble fraction and starch granule-
bound fraction by the method of Denyer et al (1995). Thus
this cDNA clone encoded a polypeptide comprising a 41 amino
acid transit peptide and a 606 amino acid mature peptide
(SEQ ID NO:12). The cleavage site LRRL was located at amino

10   acids 36 to 39 of the transit peptide of this deduced
polypeptide.

        Comparison of wheat SSS I with rice SSS and potato
SSS showed that there is 87.4% or 75.9% homology at the
amino acid level and 74.7% or 58.1% homology at the

15   nucleotide level. Some amino acids in the at N-terminal
sequences of the SSS I of wheat and rice were conserved.
Major features of the SSS I gene are summarized in Table 3.

Example 19   Isolation of Genomic Clone of Wheat Soluble
20   Starch Synthase

        Seven genomic clones were obtained with a 300 bp
cDNA probe by screening approximately 5×10^5 plaques from a
genomic DNA library of Triticum tauschii, as described
above. DNA was purified from 5 of these clones and digested

25   with BamHI and SacI. Southern hybridization analysis using
the 300 bp cDNA as probe showed that these clones could be
classified into two classes, as shown in Figure 17. One
genomic clone, sg3, contained a long insert, and was
digested with BamHI or SacI and subcloned into pBluescript

30   KS+ vector.

Table 3
Comparison of exons and introns of soluble starch synthases I genes of wheat and rice
(1) Identity of exons of soluble starch synthase I genes of wheat and rice

Exons	wSSI-D1	rSSI	identity (%)	start site (wSSI-D1)	stop site (wSSI-D1)
1a	255	113	57.52	-253	0
1b	316	298	58.92	1	316
2	356	356	82.87	1473	1828
3	78	78	92.31	2746	2823
4	125	125	90.40	2906	3028
5	82	82	89.02	4113	4194
6	174	174	93.10	4286	4459
7	82	82	93.90	4562	4643
8	92	92	92.39	4743	4835
9	63	63	90.48	4959	5021
10	90	90	82.22	5103	5192
11	125	125	88.80	8594	8718
12	109	109	91.74	8807	8915
13	53	53	81.13	8992	9044
14	40	41	80.00	9160	9199
15a	159	113	79.65	9499	9657
15b	392	539	46.46	9658	10098

(2)   Identity of introns of soluble starch synthase I genes of wheat and rice

Introns	wSSI-D1	rSSI	identity (%)	start site (wSSI-D1)	stop site (wSSI-D1)
1	1156	907	41.05	317	1472
2	917	851	41.65	1829	2745
3	82	87	45.12	2824	2905
4	1084	835	48.50	3029	4112
5	91	96	57.78	4195	4285
6	102	189	52.48	4460	4561
7	99	96	52.08	4644	4742
8	123	110	45.46	4836	4958
9	81	78	58.97	5022	5102
10	3401	663	37.56	5193	8593
11	88	124	56.82	8719	8806
12	76	81	48.68	8916	8991
13	115	135	45.22	9045	9159
14	299	830	45.80	9200	9498

Note: Exon 1a: non-coding region of exon 1. Exon 1b: coding region of exon 1.

Exon 15a: coding region of exon 15. Exon 15b: non-coding region of exon 15.

wSSI-D1: wheat soluble starch synthase I gene.

rSSI: rice soluble starch synthase I gene.

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These subclones were analysed by sequencing. The intron/exon structure of the sg3 rice gene is shown in Figure 18. The SSS I gene from *T. tauschii* is shown in SEQ ID No:13, while the deduced amino acid sequence is shown in
5   SEQ ID NO:14.

Example 20   Northern Hybridization Analysis of the
                    Expression of Genes Encoding Soluble Starch
                    Synthase
10   Total RNAs were purified from leaves, pre-anthesis material, and various stages of developing endosperm at 5-8, 10-15 and 18-22 days post anthesis. Northern hybridization analysis showed that mRNAs encoding wheat SSS I were specifically expressed in developmental endosperm.
15   Expression of this mRNAs in the leaves and pre-anthesis materials could not be detected by northern hybridization analysis under this experimental condition. Wheat SSS I mRNAs started to express at high levels at an early stage of endosperm, 5-8 days post anthesis, and the expression level
20   in endosperm at 10-15 days post anthesis, was reduced. These results are summarized in Figure 9a and Figure 9d.

Example 21   Genomic Localisation of Wheat Soluble Starch
                    Synthase
25   DNA from chromosome engineered lines was digested with the restriction enzyme BamHI and blotted onto supported nitrocellulose membranes. A probe prepared from the 3' end of the cDNA sequence, from positions 2345 to 2548, was used to hybridise to this DNA. The presence of a specific band
30   was shown to be associated with the presence of chromosomes 7A (Figure 19). These data demonstrate location of the SSS I gene on chromosome 7.

Example 22   Isolation of SSS I Promoter
35   We have isolated the promoter that drives this pattern of expression for SSS I. The pattern of expression for SSS I is very similar to that for SBE II: the SSS I gene

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transcript is detectable from an early stage of endosperm development until the endosperm matures. The sequence of this promoter is given in SEQ ID No:15.

5   Example 23   Isolation of the Gene Encoding Debranching
                       Enzyme from Wheat

The *sugary*-1 mutation in maize results in mature dried kernels that have a glassy and translucent appearance; immature mature kernels accumulate sucrose and other simple
10   sugars, as well as the water-soluble polysaccharide phytoglycogen (Black *et al*, 1966). Most data indicates that in *sugary-1* mutants the concentration of amylose is increased relative to that of amylopection. Analysis of a particular *sugary-1* mutation (*su-1Ref*) by James *et al*,
15   (1995) led to the isolation of a cDNA that shared significant sequence identity with bacterial enzymes that hydrolyse the α 1,6-glucosyl linkages of starch, such as an isoamylase from *Pseudomonas* (Amemura *et al*, 1988), _ie_. bacterial debranching enzymes.
20   We have now isolated a sequence amplified from wheat endosperm cDNA using the polymerase chain reaction (PCR). This sequence is highly homologous to the sequence for the *sugary* gene isolated by James *et al*, (1995). This sequence has been used to isolate homologous cDNA sequences
25   from a wheat endosperm library and genomic sequences from *Triticum tauschii*.
         Comparison of the deduced amino acid sequences of DBE from maize with spinach (Accession SOPULSPO, GenBank database), *Pseudomonas* (Amemura *et al*, 1988) and rice
30   (Nakamura *et al*, 1997) enabled us to deduce sequences which could be useful in wheat. When these sequences were used as PCR amplification primers with wheat genomic DNA a product of 256 bp was produced. This was sequenced and was compared to the sequence of maize *sugary* isolated by James *et al*,
35   (1995). The results are shown in Figure 20a and Figure 20b. This sequence has been termed wheat debranching enzyme sequence I (WDBE-I).

        WDBE-1 was used to investigate a cDNA library
constructed from wheat endosperm (Rahman *et al*, 1997)
enables us to isolate two cDNA clones which hybridise
strongly to the WDBE-I probe. The nucleotide sequence of
5   the DNA insert in the longest of these clones is given in
SEQ ID No:16.

        Use of WDBE 1 to investigate a genomic library
constructed from *T. tauschii*, as described above has led to
the isolation of four genomic clones, designated I1, I2, I3
10   and I4, respectively, which hybridised strongly to the
WDBE-I sequence. These clones were shown to contain copies
of a single debranching enzyme gene. The sequence of one of
these clones, I2, is given in SEQ ID No:17. The intron/exon
structure of the gene is shown in Figure 20c. Exons 1 to 4
15   were identified by comparison with the maize *sugary*-1 cDNA,
while Exons 5 to 18 were identified by comparison with the
cDNA sequence given in SEQ ID No:16. The major features of
the DBE I gene are summarized in Table 4.

        Hybridization of WDBE-I to DNA from *T. tauschii*
20   indicates one hybridizing fragment (Figure 21a). The
chromosomal location of the gene was shown to be on
chromosome 7 through hybridisation to nullisomic/tetrasomic
lines of the hexaploid wheat cultivar Chinese Spring
(Figure 21b).

25   We have clearly isolated a sequence from the wheat
genome that has high identity to the debranching enzyme cDNA
of maize characterised by James *et al* (1997). The isolation
of homologous cDNA sequences and genomic sequences enables
further characterisation of the debranching enzyme cDNA and
30   promoter sequences from wheat and *T. tauschii*. These
sequences and the WDBE I sequences shown herein are useful
in the manipulation of wheat starch structure through
genetic manipulation and in the screening for mutants at the
equivalent *sugary* locus in wheat.
35   Figure 9e shows that the DBE I gene is expressed
during endosperm development in wheat and that the timing of
expression is similar to the SBEII and SSSI genes. Figure 9h

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shows that the full length mRNA for the gene (3.0 kb) is found only in the wheat endosperm.

Example 24   Transient assays of Promoter-GFP Fusions
5 **DNA constructs**

         DNA constructs for transient expression assays were prepared by fusing sequences from the BEII and SSI promoters to the gene encoding the Green Fluorescent Protein. Green Fluorescent Protein (GFP) constructs
10 contained the GFP gene described by Sheen et al. (1995). The nos 3' element (Bevan et al., 1983) was inserted 3' of the GFP gene. The plasmid vector (pWGEM_NZfp) was constructed by inserting the NotI to HindIII fragment from the following sequence:

15 5' GCGGCCGCTC CCTGGCCGAC TTGGCCGAAG CTTGCATGCC TGCAGGTCGA CTCTAGAGGA TCCCCGGGTA CCGAGCTCGA ATTCATCGAT GATATCAGAT CCGGGCCCTC TAGATGCGGC CGCATGCATA AGCTT 3'

20 into the *NotI* and *HindIII* sites of pGem-13Zf(-) vector (Promega). The sequences at the junction of the wSSSIpro1 and wSSSIpro2 and GFP were identical, and included the junction sequence:

25 5'....CGCGCGCCCA CACCCTGCAG GTCGACTCTA GAGGATCCAT GGTGAGCAAG 3'.

The sequence at the junction of wsbeIIpro1 and GFP was:

30 5' GCGACTGGCT GACTCAATCA CTACGCGGGG ATCCATGGTG AGCAAGGGCG 3'.

The sequence at the junction of wsbeIIpro2 and GFP was:

5' GGACTCCTCT CGCGCCGTCC TGAGCCGCGG ATCCATGGTG AGCAAGGGCG
35 3'.

The structures of the constructs are shown in Figures 22a to 22f.

Table 4
Structural features of wDBEI-D1

A.
Position
of exons

Exon number	Start position	End position	Comments
1	1890	2241	(deduced by comparison with maize)
2	2342	2524	(deduced by comparison with maize)
3	2615	2707	(deduced by comparison with maize)
4	3016	3168	(deduced by comparison with maize)
5	3360	3436	
6	4313	4454	
7	4526	4633	
8	4734	4819	
9	5058	5129	
10	5202	5328	
11	5558	5644	
12	6575	6671	
13	7507	7661	
14	8450	8527	
15	8739	8823	
16	8902	8981	
17	9114	9231	
18	Still being sequenced		

5   Note that following nucleotides 3330, 6330 and 8419 there
may be short regions of DNA not yet sequenced.

B.
CAAAAT motif   1833
10   TCAAT motif   1838
ATAAATAA motif   1804

Endosperm box like motif TAAAACG   1463

Preparation of target tissue

All explants used for transient assay were from
the hexaploid wheat cultivar, Milliwang. Endosperm (10 - 12
days after anthesis), embryos (12 - 14 days after anthesis)
5   and leaves (the second leaf from the top of plants
containing 5 leaves) were used. Developing seed or leaves
were collected, surface sterilized with 1.25% w/v sodium
hypochlorite for 20 minutes and rinsed with sterile
distilled water 8 times. Endosperms or embryos were
10   carefully excised from seed in order to avoid contamination
with surrounding tissues. Leaves were cut into 0.5 cm x 1
cm pieces. All tissues were aseptically transferred onto
SD1SM medium, which is an MS based medium containing 1 mg/L
2,4-D, 150 mg/L L-asparagine, 0.5 mg/L thiamine, 10 g/L
15   sucrose, 36 g/L sorbitol and 36 g/L mannitol. Each agar
plate contained either 12 endosperms, 12 embros or 2 leaf
segments.

Preparation of gold particles and bombardment
20   Five µg of each plasmid was used for the
preparation of gold particles, as described by Witrzens *et
al*. (1998). Gold particle-DNA suspension in ethanol (10 µl)
was used for each bombardment using a Bio-Rad helium-driven
particle delivery system, PDS-1000.
25

GFP assay
The expression of GFP was observed after 36 to 72
hours incubation using a fluorescence microscope. Two plates
were bombarded for each construct. The numbers of expressing
30   regions were recorded for each target tissue, and are
summarized in Table 5. The intensity of the expression of
GFP from each of the promoters was estimated by visual
comparison of the light intensity emitted, and is summarized
in Table 6.
35   The DNA construct containing GFP without a
promoter region (pZLGFPNot) gave no evidence of transient
expression in embryo (panel 1) or leaf (panel r) and

extremely weak and sporadic expression in endosperm (panel
f) , this construct gave only very weak expression in
endosperm with respect to the number (Figure 5) and
intensity (Figure 6) of transient expression regions. The
5   constructs pwsssIpro1gfpNOT (panels b, h and n),
psbeIIpro1gfpNOT(panels d, j and p), and psbeIIpro2gfpNOT
(panels e, k and q) yielded low numbers (Table 5) of
strongly (Table 6) expressing regions in leaves, and there
was a very uneven distribution of expressing regions between
10   target leaf pieces (Table 5). pwsssIpro2gfpNOT (panels c, i
and o) gave no evidence of transient expression in leaves
(Table 5). These results show that each of the promoter
constructs is able to drive the transient expression of GFP
in the grain tissues, endosperm and embryo. The ability of
15   the short SSI promoter (pwsssIpro2gfpNOT containing 1042 bp
5' of the ATG translation start site) to drive expression in
leaves (panel n) contrasts with the inability of the long
SSI promoter (pwsssIpro2gfpNOT containing 3914 base pair
region 5' of the ATG translation start site, panel o))
20   suggesting that regions for controlling tissue specificity
are located between -3914 and -1042 of the SSI promoter
region (SEQ ID No:15).

Example 25   Stable transformation of rice
25   Stable transformation of rice using *Agrobacterium*
was carried out essentially as described by Wang et al.
1997. The plasmids containing the target DNA constructs
containing the promoter-reporter gene fusions are shown in
Figure 23. These plasmids were transformed into
30   *Agrobacterium tumefaciens* AGL1 by electroporation.and
cultured on selection plates of LB media containing
rifampicillin (50 mg/L) and spectinomycin (50 mg/L) for 2 to
3 days, and then gently suspended in 10 ml NB liquid medium
containing 100 μM acetosyringone and mixed well. Embryogenic
35   rice calli (2 to 3 months old) derived from mature seeds
were immersed in the *A. tumefaciens* AGL1

Table 5

Transient Assay of GFP based constructs

Tissue	Construct	Plate No.	1	2	3	4	5	6	7	8	9	10	11	12	Ave.	S.D.
								Explant Number								
Endosperm	pact_jsgfg_nos	1	0	0	1	158	152	148	0	2	12	159	95	64	65.9	71.6
Endosperm	pact_jsgfg_nos	2	3	13	2	83	18	9	6	188	0	102	5	3	36.0	58.6
Embryo	pact_jsgfg_nos	3	97	79	77	101	121	176	89	129	139	212	131	138	124.1	40.1
Embryo	pact_jsgfg_nos	4	18	39	89	82	7	52	94	147	19	66	106	85	67.0	41.6
Leaf	pact_jsgfg_nos	5	0	2	0	3	0	0							0.8	1.3
Leaf	pact_jsgfg_nos	6	0	0	0	1	0	0							0.2	0.4
Leaf	pact_jsgfg_nos	7	3	0	0	2	0	3							1.3	1.5
Endosperm	pZLGFPNot	8	13	0	4	0	14	0	0	0	0	0	0	1	2.7	5.2
Endosperm	pZLGFPNot	9	0	0	0	0	14	0	0	5	3	4	6	0	2.7	4.2
Embryo	pZLGFPNot	10	0	0	0	0	0	0	0	0	0	0	0	0	0.0	0.0
Embryo	pZLGFPNot	11	0	0	0	0	0	0	0	0	0	0	0	0	0.0	0.0
Leaf	pZLGFPNot	12	0	0	0	0	0	0							0.0	0.0
Leaf	pZLGFPNot	13	0	0	0	0	0	0							0.0	0.0
Leaf	pZLGFPNot	14	0	0	0	0	0	0							0.0	0.0

Table 5 (Continued)

Transient Assay of GFP based constructs

Tissue	Construct	Plate No.	Explant Number												Ave.	S.D.
Endosperm	psbeIIpro1gfpNOT	15	111	0	77	142	0	127	7	35	39	191	95	34	71.5	62.3
Endosperm	psbeIIpro1gfpNOT	16	21	101	0	0	34	164	102	5	39	125	147	114	71.0	60.6
Embryo	psbeIIpro1gfpNOT	17	23	67	63	4	12	14	9	8	29	19	24	51	26.9	21.7
Embryo	psbeIIpro1gfpNOT	18	92	144	64	36	31	23	106	43	11	1	9	7	47.3	45.4
Leaf	psbeIIpro1gfpNOT	19	0	0	0	0	0	0							0.0	0.0
Leaf	psbeIIpro1gfpNOT	20	6	0	0	0	0	0							1.0	2.4
Leaf	psbeIIpro1gfpNOT	21	0	0	0	0	3	5							1.3	2.2
Endosperm	psbeIIpro2fpNOT	22	12	18	3	0	0	21	13	0	10	11	10	0	8.2	7.4
Endosperm	psbeIIpro2fpNOT	23	24	25	13	68	11	0	0	0	1	0	0	0	11.8	20.1
Embryo	psbeIIpro2fpNOT	24	9	13	4	7	6	21	0	9	3	5	2	4	6.9	5.7
Embryo	psbeIIpro2fpNOT	25	5	0	3	5	23	4	3	1	8	12	8	13	7.1	6.4
Leaf	psbeIIpro2fpNOT	26	0	2	0	0	0	0							0.3	0.8
Leaf	psbeIIpro2fpNOT	27	0	5	0	8	0	0							2.2	3.5
Leaf	psbeIIpro2fpNOT	28	0	0	0	0	0	0							0.0	0.0

Table 5 (Continued)

Transient Assay of GFP based constructs

Tissue	Construct	Plate No.	Explant Number												Ave.	S.D.
Endosperm	pwsssIpro1gfpNOT	29	121	0	0	28	0	4	81	23	0	2	0	2	21.8	39.2
Endosperm	pwsssIpro1gfpNOT	30	3	0	0	92	12	0	0	102	4	159	41	24	36.4	52.8
Embryo	pwsssIpro1gfpNOT	31	112	106	74	54	33	73	77	49	42	38	59	46	63.6	25.6
Embryo	pwsssIpro1gfpNOT	32	97	48	110	22	191	112	53	6	9	145	6	10	67.4	62.4
Leaf	pwsssIpro1gfpNOT	33	0	0	0	0	0	0							0.0	0.0
Leaf	pwsssIpro1gfpNOT	34	0	0	0	0	0	0							0.0	0.0
Leaf	pwsssIpro1gfpNOT	35	12	0	0	0	0	0							2.0	4.9
Endosperm	pwsssIpro2fpNOT	36	0	0	18	81	0	0	0	6	0	0	1	0	8.8	23.3
Endosperm	pwsssIpro2fpNOT	37	0	18	14	6	63	8	8	23	79	7	46	51	26.9	26.1
Embryo	pwsssIpro2fpNOT	38	15	7	14	57	8	3	26	10	47	34	47	0	22.3	19.4
Embryo	pwsssIpro2fpNOT	39	9	15	48	103	31	22	107	22	27	82	51	63	48.3	33.8
Leaf	pwsssIpro2fpNOT	40	0	0	0	0	0	0							0.0	0.0
Leaf	pwsssIpro2fpNOT	41	0	0	0	0	0	0							0.0	0.0
Leaf	pwsssIpro2fpNOT	42	0	0	0	0	0	0							0.0	0.0

Table 6
Comparison of the Intensities of Transient Expression

Tissue	pact_js-gfg_nos	pwsssI-pro1gf pNOT	pwsssI-pro2gf pNOT	psbeII-pro1gf pNOT	psbeII-pro2gf pNOT	pZLGFP Not
Endosperm	10	4	2.5	3.5	1.5	0.5
Embryo	10	5.5	5.5	1.5	1	0
Leaf	10	20	0	10	10	0

5   All intensities are relative to pact_js-gfg_nos transient
expression in the target tissue
Relative intensities were independently scored by three
researchers and averaged.

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suspension. After 3 - 10 minutes the A. tumefaciens AGL1
suspension medium was removed, and the rice calli were
transferred to NB medium containing 100 μM acetosyringone
for 48 h. The co-cultivated calli were washed with sterile
5   Milli Q H$_2$O containing 150 mg/L timentin 7 times to remove
all Agrobacterium, plated on to NB medium containing 150
mg/L timentin and 30 mg/L hygromycin, and cultured for 3 to
4 weeks. Newly-formed buds on the surface of rice calli were
excised and plated onto NB Second Selection medium
10   containing 150 mg/L timentin and 50 mg/L hygromycin. After 4
weeks of proliferation calli were plated onto NB Pre-
Regeneration medium containing 150 mg/L timentin and 50 mg/L
hygromycin, and cultured for 2 weeks. The calli were then
transferred on to NB-Regeneration medium containing 150 mg/L
15   timentin and 50 mg/L hygromycin for 3 to 4 weeks. Once
shooting occurs, shoots are transferred onto rooting medium
(½ MS) containing 50 mg /L hygromycin. Once adequate root
formation occurs, the seedlings are transferred to soil,
grown in a misting chamber for 1-2 weeks, and grown to
20   maturity in a containment glasshouse.

Example 26   Use of probes from SSS I, SBE I, SBE II and
                  DBE sequences to identify null or altered
                  alleles for use in breeding programmes
25   DNA primer sets were designed to enable
amplification of the first 9 introns of the SBE II gene
using PCR. The design of the primer sets is illustrated in
Figure 24. Primers were based on the wSBE II-D1 sequence
(deduced from Figure 13b and Nair et al, 1997; Accession No.
30   Y11282) and were designed such that intron sequences in the
wSBE II sequence were amplified by PCR. These primer sets
individually amplify the first 9 introns of SBE II. One
primer (sr913F) contained a fluorescent label at the 5' end.
Following amplification, the products were digested with the
35   restriction enzyme DdeI and analysed using an ABI 377 DNA
Sequencer with Genescan™ fragment analysis software. One
primer set, for intron 5, was found to amplify products from

each of chromosomes 2A, 2B and 2D of wheat. This is shown in
Figure 25, which illustrates results obtained with various
wheat lines, and demonstrates that products from each of the
wheat genomes from diverse wheats were amplified, and that

5   therefore lines lacking the wSBEII gene on a specific
chromosome could be readily identified. Lane (iii)
illustrates the identification of the absence of the A
genome wSBEII gene from the hexaploid wheat cultivar Chinese
Spring ditelosomic line 2AS.

10   Figure 26 compares results of amplification with
an Intron 10 primer set for various nullisomic/tetrasomic
lines of the hexaploid wheat Chinese Spring. Fluorescent
dUTP deoxynucleotides were included in the amplification
reaction. Following amplification, the products were

15   digested with the restriction enzyme DdeI and analysed using
an ABI 377 DNA Sequencer with Genescan™ fragment analysis
software. In lane (i) Chinese Spring ditelosomic line 2AS, a
300 base product is absent; in lane (ii) N2BT2A, a 204 base
product is absent, and in lane (iii) N2DT2B a 191 base

20   product is absent. These results demonstrate that the
absence of specific wSBEII genes on each of the wheat
chromosomes can be detected by this assay. Lines lacking
wSBEII forms can be used as a parental line for breeding
programmes for generation of new lines in which expression

25   of SBE II is diminished or abolished, with consequent
increase in amylose content of the wheat grain. Thus a high
amylose wheat can be produced.

            Table 7 shows examples primers pairs for SBE I,
SSS I and DBE I which can identify genes from individual

30   wheat genomes and could therefore be used to identify lines
containing null or altered alleles. Such tests could be used
to enable the development of wheat lines carrying null
mutations in each of the genomes for a specific gene (for

Table 7

PCR Primers for Starch Biosynthesis Genes

Gene	Foward Primer	Foward Primer sequence	Reverse Primer	Reverse Primer sequence	Temp (°C)	Product (bp)
SBE I	ZLE1 5d	GGC GGC GGC AAT GTG CGG CTG AG	ZLBE1 63	CCA GAT CGT ATA TCG GAA GGT CG	57.3	A=625, B = 600, D = 550
SSS I	sssE01F	GAA CTC GCG CCC GAC CTC CT	ZLSg7	AGC CAC GAT TAT GCT GTC GAT GG	55.0	A, 450; B=450; D= 630
	sssE14F	TTC TCA CCG CTA ACC GTG GAC	ZLSm19	GTC TAC ATG ACG TAG GGT TGG TC	55.8	B = 400, D = 500 no A product
DBE I	DBEE17F	TGG TCT GAG AAT AGC CGA TTC	sr1536F	AAGGCCACATAGATCTCG	56.8	B, 190; D, 190, A, 160. Non-specific product 220 bp

Temp: = annealing temperature, bp = length of the product in base pairs

example SBEI, SSI or DBE I) or combinations of null alleles
for different genes.

It will be apparent to the person skilled in the
art that while the invention has been described in some
5   detail for the purposes of clarity and understanding,
various modifications and alterations to the embodiments and
methods described herein may be made without departing from
the scope of the inventive concept disclosed in this
specification.

10   Reference cited herein are listed on the following
pages, and are incorporated herein by this reference.

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